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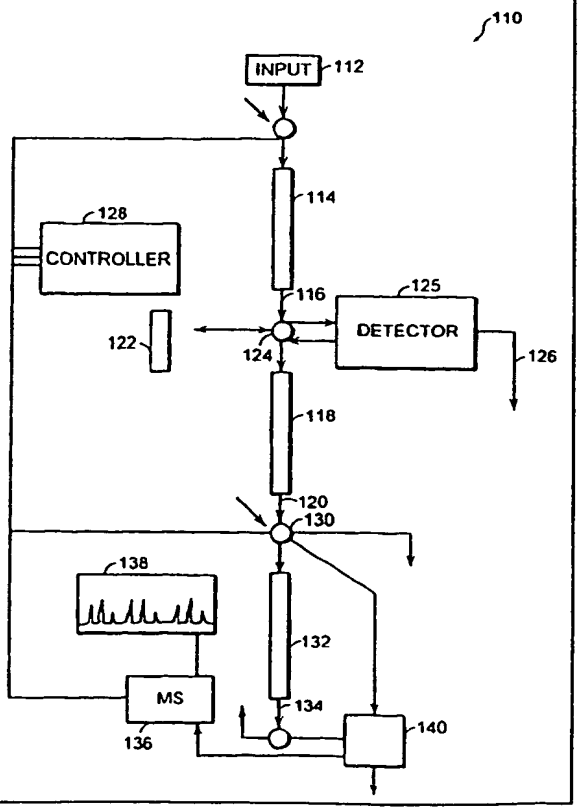
(57) Abstract

The invention provides novel methods for screening a sample to select a ligand to a target of interest and for obtaining information about the ligand and its binding characteristics. Specifically, the claimed multi-dimensional methods involve combining a solution of heterogeneous ligands with the target of interest to screen the ligands on the basis of one or more binding characteristics. Ligands having the first binding characteristic bind to the target of interest thereby to form a target/ligand complex. The complex then optionally is separated from the unbound components using any of a variety of separation techniques, e.g., size exclusion. At least one of the complex or unbound components then is introduced to a second "dimension". The second dimension is capable of separating components based upon a second binding characteristic. One then elutes the ligand having the desired binding characteristics.



HIGH SPEED, AUTOMATED, CONTINUOUS FLOW, MULTI-DIMENSIONAL MOLECULAR SELECTION AND ANALYSIS

RELATED APPLICATION

This application is based on provisional application serial number 60/000,518 filed June 26, 1995, the disclosure of which in its entirety is herein incorporated by reference.

FIELD OF THE INVENTION

This invention relates to the integrated, multi-dimensional, rapid analysis of solutions of a large number of mixed molecular species, commonly called "libraries". More specifically, the invention is directed toward methods for the discovery of molecular entities useful in a variety of biological contexts using hi-flux screening of natural and synthetic libraries to select ligands having a desired affinity for a target molecule of interest.

BACKGROUND

Multi-dimensional systems, i.e., systems involving the application of multiple distinct physico-chemical separation steps, are known to be useful for many applications. The purification of proteins, for example, frequently is accomplished using multiple passes through different chromatographic columns exploiting differential partitioning such as adsorption and size exclusion. Inherent in any multi-dimensional process is the necessity to identify the desired component, from the output of the first partition, to collect it, and to introduce it into the next dimension of the system. Disadvantages of such systems include slow analysis, solvent incompatibility between successive partitioning phases, the necessity of labor intensive handling, and consequent contamination or loss of sample.

The recent prior art discloses various new methods for implementing the search for novel agents such as, for example, pharmacological or therapeutic agents (i.e., drug discovery) agents useful in animal care or management, agriculturally useful chemicals, selective biocides for insects, weeds, or other pests, and catalytic and other entities useful in industrial processes. Collections of molecules or "libraries" are prepared and screened for molecules having a specified bioactivity, as

The screening methods described immediately above are based upon identifying which ligand in a mixture binds to a target of interest. Binding typically is assayed with either the ligands of the library or the target immobilized on some form of solid support. Various solution parameters may be adjusted to emulate different binding conditions and to obtain different ligands. Often, peptides which are obtained through procedures involving their immobilization to a support have disappointing affinity, i.e., have a binding constant too low to be useful. Traditionally, antibodies are used for the affinity purification of proteins and other biomolecules. However, the cost of generating antibodies, the potential for antibody leaching, and the need for relatively harsh eluting conditions pose problems for the routine use of antibodies in affinity purification.

Screening methods known in the art thus are not entirely satisfactory. Prior methods for detecting or identifying ligands which bind to a target of interest often fail to provide ligands of sufficiently high affinity to be useful, and additionally suffer from the loss of sample, the need for large amounts of ligands, and the need to vary loading, binding, or elution conditions to obtain useful results. Additionally, existing systems are unable selectively to screen a library while simultaneously determining the affinity of selected ligand(s) for the target under relevant conditions.

A major hurdle in the exploitation of current screening techniques of the type described above is effective chemical characterization of ligands identified in these processes. Chemical characterization, e.g., determining the sequence of an identified biopolymer, is at best time-consuming and complex. A major focus of prior art screening techniques is to enable the collection of enough of or enough information about a ligand of interest so as to permit determination of its structure and to enable synthesis of larger amounts for testing and further empirical structural refinement.

Accordingly, there is a need for integrated, multi-dimensional screening, selection and analysis systems and methods which permit automated, direct transfer of samples without dilution or loss between various dimensions, and efficiently screen for, and subsequently permit

SUMMARY OF THE INVENTION

Accordingly, the present invention is directed to rapid, efficient and automated, multi-dimensional systems, methods and apparatus for screening libraries to select, recover and characterize a candidate ligand with a desired or preselected affinity K for a preselected target molecule. Additionally, the present invention is directed to certain combination of individual dimensions of such a system, which can be used to obtain a desired result, and, specifically to a method of detecting a ligand to a target of interest which overcomes the disadvantages of the methods known in the art.

Additional features and advantages of the invention will be set forth in the description which follows, and in part will be apparent from the description and drawing, or may be learned by practice of the invention. The objectives and other advantages of the invention will be realized and attained by the process particularly pointed out in the written description, drawing, and appended claims.

To achieve these and other advantages, and in accordance with the invention as embodied and broadly described, the invention provides novel methods for screening a sample to select a ligand to a target of interest and for obtaining information about the ligand and its binding characteristics. Specifically, the claimed multi-dimensional methods involve combining a solution of heterogeneous ligands with the target of interest to screen the ligands on the basis of one or more binding characteristics. Ligands having the first binding characteristic will bind to the target of interest to form a target/ligand complex. The complex then optionally is separated from the unbound components using any of a variety of separation techniques, e.g., size exclusion. At least one of the complex or unbound components then is introduced to a second "dimension". The second dimension is capable of separating components based upon a second binding characteristic. One then elutes the ligand having the desired binding characteristics.

Additionally, the invention relates to a method of detecting the presence of a ligand having a desired or preselected affinity (K) for a preselected target molecule in a sample of ligands in a solvent by loading a column with a known concentration of target molecules (T), and passing the sample through the column so that ligands in the sample bind to the column through the target

mixed bed ion exchanger, a cation exchanger or an anion exchanger, and means to inject solvent so that the pH, ionic strength, etc. can be controlled so as to permit further downstream partitioning of partly screened ligand species.

The ability of the methods and apparatus of the invention to provide for continuous flow through multiple partitioning dimensions is dependent in many cases on the use of interface columns. These condition the solvent containing the dissolved ligands exiting an upstream column for effective partitioning in a downstream column. In one such interface, effluent high in salt is desalted by passage through a reverse phase column. The ligands adsorb, the salt is washed out, and the ligands then are eluted with salt-free or low salt solvent. In another, organic solvent such as acetonitrile is removed by passing the solution through an ion exchange column, binding the ligands therein, and subsequently eluting with an aqueous eluant. In still another, the pH of acidic solvents is increased by binding in a cation exchange resin, washing out the acid, and eluting in, e.g., a neutral pH solvent. Similarly, the pH of alkaline solvents may be decreased by binding in an anion exchange resin.

In yet other embodiments, the invention features an interface for sampling a liquid chromatographic (LC) exit stream, and delivering the sample to a mass spectrometer (MS). The sampler has a predetermined sample volume disposed, for example, in a sample loop, alternatively switchable to extract from an LC exit stream, and to insert into an analysis stream of an MS. A sample controller cycles the sampler to first extract and then to insert the sample. In various embodiments, the sampler can comprise a multi-port valve and the sample volume is disposed within tubing of a predetermined volume. The sample controller may cycle the sampler to take a sample of the LC eluate a plurality of times during an LC analysis peak. Other embodiments may include a second sampler. The first and second samplers can be placed in series.

The embodiments of the methods, apparatus and system of the invention described above may optionally include a detector for identifying a selected ligand. The detector may consist of, for example, a mass spectrometer or a fluorescence detector.

Additionally, in other embodiments, the invention relates to a method of detecting a ligand having a desired high affinity K for a preselected target molecule when the ligand and the target

thereafter the ligands which bind to the target (forming a complex) are separated from those which do not bind. The sample solutions may be obtained by the digestion of any protein, including post-translationally modified proteins, antibodies, etc.

The methods of the claimed invention also relate to detecting a ligand in a library which will bind when the ligand and the target molecule are present together in preselected solvent conditions, e.g., physiological saline. As before, a target molecule is immobilized onto a column, and the sample is passed through the column under the preselected solvent conditions. Next, a series of column volumes of solvent is passed through the column to select a desired ligand. The eluate is then introduced to a ligand accumulator.

The methods also relate to the preparation of pharmaceutically active compositions using the multi-dimensional methods described above, and to the subsequent commercial production of such compositions.

In yet other aspects, the invention relates to methods of selecting ligands based upon one or more binding characteristics by the use of multiple dimensions.

In an important aspect, the invention provides apparatus and methods which are automated, fast, and operate by continuous flow. The methods are capable in preferred embodiments of selecting ligands having affinity and specificity for essentially any target molecule, separating the members of the select group from one another, and obtaining physico-chemical data characteristic of the structure of the selected ligands. The nature of the library useful in the system essentially is unlimited. Thus, mixtures of organic compounds may be used. Digests of biopolymers, either natural or synthetic, are particularly attractive. Such digests may comprise mixtures of peptides, polysaccharides, polynucleotides, various derivatized forms thereof, and variously sized fragments thereof. The biopolymers may be extracted from plant or animal tissues, diseased or healthy, digested if necessary, or used as is. Such libraries are available in abundance, easy to prepare, may be of lower toxicity and more stable than synthetic peptides, and may be varied and screened systematically.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic representation of a splitter interface (sampler) between a liquid chromatography column and a mass spectrometer in the apparatus of the invention (Position A).

Fig. 2 is a schematic representation of a splitter interface (sampler) between a liquid chromatography column and a mass spectrometer in the apparatus of the invention (Position B).

Fig. 3 is a schematic representation of one embodiment of the apparatus of the invention.

Fig. 4 is a schematic representation of a second embodiment of the apparatus of the invention.

Fig. 5 is a diagram of a BIOCAD™ Workstation available from PerSeptive Biosystems, Inc. which is plumbed in tandem column mode. Column 1 is a weak anion exchange column and column 2 is a reversed phase column.

Fig. 6 depicts target-based screening of human rHsp70.

Figs. 7A, 7B, 7C and 7D depict MALDI spectra of natural peptide library screen vs. rHsp70 and Dnak. Fig. 7A depicts the MALDI spectrum of a rHsp70 sample incubated with the PDL. Fig. 7B depicts the MALDI spectrum of a control incubation with rHsp70 alone. Fig. 7C depicts the MALDI spectrum of a control incubation with PDL alone. Fig. 7D depicts the MALDI spectrum of Dnak incubated with PDL.

Fig. 8 identifies peptide sequences binding to Concanavalin A in a sugar-specific manner.

Mass spectrometer - a machine adapted for the introduction of microsamples containing a selected ligand or fragments of a ligand, or which generates ligand fragments, and measures the mass to charge ratio of solutes in the sample to provide data helpful or sufficient to determine the structure of the ligand.

MS/MS - a mass spectrometer detector of the type that determines the mass to charge ratio both of a ligand inserted therein and then of fragments of that ligand generated by ionization or otherwise.

Reverse Phase - a chromatography surface characterized by an abundance of hydrophobic moieties.

Target Molecule - a compound such as a receptor, enzyme, DNA, RNA, etc. comprising either a) the moiety to which a selected ligand will bind with at least some selectively and reasonably high affinity, i.e., is the molecule which will be exploited during use of the selected ligand, or b) is a moiety which the selected ligand is selected specifically not to bind, so as to avoid cross reactivity or the like.

I. NATURE OF LIBRARY

A "library" as used herein encompasses virtually any solution of compounds to be screened for a ligand having an activity of interest. The library may, for example, comprise a natural or synthetic combinatorial library; solutions obtained naturally, such as from body fluids, plant fluids; or virtually any other natural or synthetic substances which can be put into solution and detected by a physical or chemical characteristic. Thus there are limitless sample possibilities and the skilled artisan can choose a sample based upon his particular application.

In various embodiments it is preferable to use a natural library of molecules obtained by the digestion of one or more natural substances. When screening for lead compounds for pharmaceutical applications (i.e. drug discovery), the sample may be obtained by the digestion of one or more molecules obtained from the host organism. It may be preferable to digest a molecule indigenous to the host which has the desired biological activity. The inventors have discovered that these libraries have a high likelihood of containing a fragment having the desired activity against a target of interest.

Natural libraries may be prepared by enzymatic digestion or other manipulation of a sample prior to screening, or in certain instances, may be a solution as found in nature without

II. THE MULTIDIMENSIONAL APPROACH

A. THEORETICAL BASIS FOR SCREENING

Peptide combinatorial libraries and natural proteolytic mixtures contain three types of peptides; those that i) have no affinity to any protein, ii) bind to a large number of proteins, or iii) show affinity to a specific protein. The later group may be further subdivided according to binding affinity and the specific site on the protein surface to which the peptide binds. It is necessary in a "screening" system to differentiate between these various peptides.

It has been noted above that protein also referred to herein as the target (R)/ligand (L) association may be described by the formula

$$[R] + [L] \text{ ------ > } [RL] \qquad (A)$$

and the equation

$$K_b = k_1/k_2 = [RL] / [R]\,[L] \qquad (1)$$

where K_b is the binding constant and the rate constants k_1 and k_2 represent the forward and reverse rate constants, respectively. The general way in which peptides from synthetic libraries are screened is i) to use an excess of peptide, ii) control the conditions of association, iii) allow the system to come to equilibrium, and iv) then rapidly separate the unbound peptides from the RL complex. From this point on the various screening systems diverge in the identification of bound peptides.

The claimed approach is quite different than that used by others. The methods described herein allow us to select on the basis of the forward rate constant of a ligand for the receptor, the reverse (off) rate constant, or the equilibrium constant under conditions where it is possible to vary ionic strength, pH, concentration of competitive binding agents, organic solvent concentration, and temperature to name a few. All of these conditions potentially impact complex (RL) formation.

Selection of peptides based on their binding constant can be achieved in several different ways. One is through the use of a chromatography column with the receptor (R) immobilized. Another is in a chromatographic system in which the components of the RL complex are separated as the complex dissociates.

concentration in the accumulated fraction. This may be achieved by using a reversed-phase chromatography column as an accumulator by coupling it in tandem with the affinity column. To determine k' by this method, multiple samples must be collected and quantitated to allow reconstruction of the chromatography peak. Assuming that the peak will always be of the same shape, k' may be estimated by determining the peak width and the fractional amount of the analyte eluted at any point in time.

The chromatography column is essentially one theoretical plate, i.e. 1-10 mm length, which is saturated with ligand (L) to form RL complex. Although substantial quantities of RL may be formed in the loading process, it is still possible that there is a finite quantity of residual receptor (R), especially as ligand elutes from the column. Elution of ligand (L) from this column depends on the dissociation process (formula B) in which

$$[RL] \text{ -------- } > [R] + [L] \qquad (B)$$

Free ligand is swept from the system before it has the chance to recomplex with R to form RL. Because the binding constant is very large, i.e. $> 10^6$, most of the ligand exists in the column in RL complex. This means that the rate of elution of ligand from the column will be described by the equation

$$d[RL] / dt = - F[L] / V_c \qquad (7)$$

where F is the volumetric flow rate (ml/min), [L] is ligand concentration, and V_c is the column volume (ml). Integration indicates that

$$\log [RL] = \{[L]F / V_c\}\log 1/t \qquad (8)$$

But we know that

$$K_b = \{[RL]_i - [L]\} / \{[R]_i + [L]\}[L] \qquad (9)$$

where K_b is the binding constant, $[RL]_i$ is the initial concentration of the RL complex, and $[R]_i$ is the initial concentration of free receptor. Substituting for [L] in the integrated form of the equation allows one to predict the rate of elution of ligand from the column as a function of the binding constant.

of ligand adsorbed to the column is $[RL]_i As$. In the special case where 50% of the ligand initially adsorbed on the column desorbs from the surface and enters the liquid phase, i.e.

$$[RL]\ As = [L]V = 1/2[RL]_i As = [R]As \tag{10}$$

then

$$K_b[L]V = 1 \tag{11}$$

In the more general case

$$K_b\{a / (1-a)\}[L]V = 1 = (K_b\{a / (1-a)\}[RL]V) / 2 \tag{12}$$

where a is the fraction of the initial adsorbed ligand that dissociates and enters the liquid phase and (1-a) is the fraction of the initial RL complex remaining after reequilibration. When two substances are bound

$$K_{b1}\{a_1 / (1-a_1)\}[RL_1]V = 1 = K_{b2}\{a_2 / (1-a_2)\}[RL_2]V = 1 \tag{13}$$

to the column and K_{b2} is the known, the equation

$$K_{b1} = K_{b2}\{a_2 / (1-a_2)\}[RL_2]\{(1-a_1) / a_1\}(1/[RL_1]) \tag{14}$$

allows one to calculate the binding constant K_{b1} based on the relative amounts of the two substances eluted from the column when the equilibrium shifts to compensate for the increase in volume (V).

As noted above, screening may also be achieved in a chromatographic system by chromatographing the RL complex and separating receptor (R) from ligand (L) as the complex RL dissociates to prevent reassociation. In this process, RL complex of those ligands with the highest binding affinity will be the most likely to survive passage through the chromatographic system without dissociation. The rate at which R is separated from L, i.e. resolution (R_s) as a function of time (dR_s / dt), is an important issue. Resolution in a chromatographic system is shown in eqn. 6, where $R_s = (V_{c2} - V_{c1}) / v$. Because dt is inversely related to mobile phase velocity (V_m),

$$dR_s / dt = V_m(V_{c2} - V_{c1}) / v \tag{15}$$

the fact that subsequent to complex dissociation there is a low probability that ligand will contact a receptor bearing surface again before elution from the system.

Mass transfer to the walls of open tubular columns eluted with aqueous mobile phases is known to be poor. Mass transfer to the walls of open tubular columns decreases with the inverse square of increasing column diameter and the square of increasing linear velocity of the mobile phase. Columns of 300 -1000 μm with immobilized receptor (R) are loaded by filling the column with a series of ligands and allowing the system to come to equilibrium at zero mobile phase velocity. When the sum of the concentration of ligand species ($[L_1]$ --- $[L_n]$) > [R], there is competition between the ligands for a binding site. The amount of any RL complex formed is a function of both the constant of the particular ligand for the receptor and the concentration of that ligand. After equilibrium has been achieved, unbound ligands are swept from the column. Elution of L in bound RL complex is based on the off-rate. Assuming that subsequent to dissociation of RL to L there is no reassociation, the rate of ligand (L) elution from the column is given by the equation

$$-d[RL] / dt = k_2[RL] \quad (20)$$

where k_2 is the off-rate constant. Integrating between the limit of the initial concentration $[RL]_i$ of complex and the concentration [RL] at time t produces the equation

$$2.3 \log ([RL] / [RL]_i) = -k_2 t \quad (21)$$

The off -rate and the rate at which ligand are eluted from the column may also be expressed in terms of the half-life ($t_{1/2}$), i.e. the time it takes for half the ligand to elute from the column. From the equation above it may be shown that

$$t_{1/2} = 0.693 / k_2 \quad (22)$$

In summary, the basic assumption in this model of off-rate selection is that there will be no reassociation of R and L at the walls of the capillary once dissociation has occurred. This probably is not strictly true. The walls of a capillary are not well swept, i.e. there is a stagnant layer of liquid at the walls. L must diffuse through this layer before it escapes into the rapidly moving liquid in the center of the capillary where it is rapidly transported out of the capillary. The validity of using this model is that mass transfer i) dominates selection processes in

B. CONFIGURATIONS

In certain embodiments, the invention may comprise a first column having the target of interest immobilized thereon. Thus, when the sample is passed over the first column, ligands which bind to the target will be immobilized thereon by forming a target/ligand complex. The complex captured by the first column is dissociated by varying the wash volumes.

The claimed invention provides a method, system and apparatus for obtaining information about a particular ligand without the need for manual manipulations, regardless of the size of the sample, or the amount of ligand present in the sample. These methods remarkably provide for the first time, rapid, automatable means for drug discovery. Literally millions of compounds can be screened for a particular biological activity in a short period of time, thus leading to significant advances in the field of biotechnology, pharmaceutical development, diagnostics and therapeutics. The methods of the invention allow the artisan to select ligands to a target of interest based upon any one or more characteristics, such as (1) the forward rate constant of a ligand for the target, i.e. on rate, (2) the reverse rate constant, i.e., off-rate, or the equilibrium constant under conditions where it is possible to vary ionic strength, pH, concentration of competitive binding agents, organic solvent concentration, and temperature, for example. Any conditions which may potentially impact the formation of a target/ligand complex may be varied and used as a selection criteria. The methods of the invention also allow the rapid selection of a ligand to a target of interest, characterization of virtually any potential binding characteristics of the ligand, and recovery of the ligand.

The methods of the invention use a tandem column chromatographic technique: any column capable of separating molecules can be used in the methods of the invention. Thus, depending on the result sought, the columns in the system may be chosen from the group consisting of affinity columns, size exclusion columns, and/or reversed phase columns. As used herein, the term "tandem mode" indicates that at least two columns are involved in the system, either simultaneously, or sequentially. If the columns are run simultaneously in tandem mode, then sample solution may be split and delivered to each column. If they are run sequentially, the

scale synthesis of the ligand or derivatives thereof for diagnostic or therapeutic applications. The most effective combination of analysis dimensions can easily be determined by one skilled in the art based upon the particular results desired.

Such multidimensional systems allow the rapid screening of libraries of ligands for their ability to bind to a certain "target molecule" ("target"or "receptor") of interest. The target molecule can be any molecule to which a ligand is desired, such as, for example, proteins, peptides, nucleic acids, monoclonal or polyclonal antibodies, etc.

Screening soluble libraries of peptides or small molecules for identification of ligands binding to a specific target molecule has become a widely used technique in the pharmaceutical industry. Screening these libraries may lead to development of novel therapeutics and/or diagnostics. Screening requires both a selection process and a method to assess the relative affinities of the ligands. Methods known in the art thus require two separate steps to determine the acceptability of a particular ligand. Often, after screening the sample and recovering a desired ligand, it is discovered that the ligand selected has an undesirable affinity for the ligand. As will be recognized by one skilled in the art, different applications often require ligands having a specific affinity. Numerous variables must be taken into account to determine the applicability of the selected ligand for the desired application. For example, one may prefer a ligand having a particular on- or off-rate, or a ligand which binds to one target molecule, but not another. Until the present invention, therefore, the entire selection process involved several separate steps, in order to not only identify a ligand to a target, but to further select based upon additional characteristics.

The claimed methods allow the novel and rapid determination of relative affinities for ligands which bind to a selected target molecule, additionally, the methods provide for the recovery of the target-bound ligands, and offers the simultaneous selection of ligands and determination of their relative affinities or other binding characteristics during the screening of a library of compounds

Multi-dimensional analysis systems as disclosed and claimed herein provide multiple embodiments suitable for the screening, selection and recovery of desired ligands. It is preferable

polysaccharides, mucopolysaccharides, antibodies or binding proteins. Additionally useful target molecules include major histocompatibility molecules, T-cell receptors, antigens, cell-adhesion molecules, cellular receptors for hormones and growth regulatory factors and virus receptors. Ligands to these targets of interest provide a collection of possible immune agonists, antagonists, antiviral ligands and structural lead compounds for the design of small molecules with a desired bioactivity. As the sample solution passes through the support, ligands to the target will adsorb at the binding sites, and be retained on the column. A certain amount of solution components may also non-specifically bind to the support.

The affinity columns to be used in the invention may comprise any solid support which does not affect the binding activity of the target molecule. Supports commonly utilized are controlled pore glass, silica, silica gel, membranes, polystyrene based beaded supports, glass fibre frits and paper filters. While perfusive matrix materials are preferred, the invention also can be practiced with a non-porous matrix, in which tortuous channels are formed by the interstitial space among non-porous packed particles. These matrices have a lower net capacity than perfusive matrices but they may be very useful for microanalysis. In addition to packed particles, matrices useful in the process and apparatus of the invention may be embodied as bundles of microcapillaries. A high surface area/volume ratio may be provided by the use of very small internal diameter capillaries, providing a reaction vessel of a few microliters/cm. Likewise, the binding protein may be coated on the inner surface of the capillary tube. Solutes may be transported through the capillary tube matrix by convection. The high surface area to volume ratio of the capillary tubes increases the available reaction volume. The matrices may further comprise a membrane structure.

The matrix preferably is a rigid substantially non-microporous, particulate material having a hydrophilic surface, and preferably is also a perfusive chromatography matrix. The matrix also may be defined by the interior surface of a capillary. The methods comprise first loading a column with a known concentration T of target molecules.

In an alternative embodiment, the first column may comprise a size-exclusion column or dialysis system, capable of separating components of the sample based on size. The different

may be preequilibrated with a ligand prior to immobilization, or prior to introduction of the soluble library.

Thus, in certain embodiments, the first affinity column having a target immobilized thereon, is plumbed, in tandem mode, to an accumulator, such as a reversed phase column to immobilize thereon ligands having the desired affinity.

The elution of the reversed-phase column allows recovery of the ligands, and the peak heights of corresponding peaks provide a measurement of the amount of the ligand bound to the target at a specific wash volume. Under these conditions, the wash volume correlates with the dissociation rate constants of the ligands, and may provide an indication of relative affinities of the ligands having a similar mode of interaction with a target. The methods can be applied to the screening of a library of compounds where selection of ligands and determination of their relative affinities can be accomplished simultaneously, enabling selection of binders with the desired affinity.

Thus, for the first time, the practitioner is able to select a ligand based not only on its ability to bind to a target, but also upon its affinity for that target, i.e. the on-rate or dissociation rate (off-rate). This ability is especially relevant in drug screening applications where the dissociation rate may be critical to the effectiveness of the composition. Since the ligands bound to the target can be later recovered, further characterization or development of the selected binders can be performed, as discussed in more detail below.

The methods allow the practitioner to select ligands to the target of interest using virtually any affinity selection method. For example, by varying the stringency, i.e. varying the wash volumes, one can to select based upon for example (1) affinity, (2) on rate or off rate; (3) the wash conditions (pH, ionic strength, temperature).

The methods allow the practitioner to select ligands to the target of interest using virtually any affinity selection method. The most effective combination of analysis dimensions can easily be determined by one skilled in the art based upon the particular results desired. The claimed system is advantageous in that the direct coupling of various unit operations with μL volume connections diminishes the dilution, loss and contamination of samples by circumventing fraction collection

the column. Obviously, the middle eluate is a combination of varying affinities for both subclass one and two.

In other embodiments, the methods and systems of the invention encompass other techniques for selecting ligands to a target of interest. The inventors have developed novel methods not only for selecting particular ligands based on affinity or subtractive screening, but also for selecting ligands which bind to a target of interest at a particular binding site. In the latter embodiments, the invention contemplates the use of a first separation system, followed by an accumulator such as a reversed phase column. As discussed above, the methods involve two dimensions, plumbed in tandem mode.

The first dimension, a size exclusion chromatography system, is very similar to a dialysis system in which the inability of a target or receptor to penetrate a pore matrix excludes it from certain liquid elements of the system. In fact, this first dimension can be a dialysis system or any other system capable of separating target/ligand complexes and unbound components. Thus, one may contact a sample solution with the target of interest, ligands to the target will bind thereto. The solution may then contain a mixture of unbound sample components and ligand/target complex which may be introduced into an SEC column. Alternatively, one may introduce the target and sample directly into the SEC column. When one introduces this mixture to an SEC system, unbound components will diffuse into the pores of the SEC matrix, however the complex, and the target are excluded because of their size. Because the macromolecular target/ligand complex moves through an SEC column faster than the lower molecular weight components, the complex will elute from the column first. The eluate containing target/ligand complexes can then be introduced to a second dimension such as an affinity column. One immobilizes a known ligand to the binding site of interest on the affinity column. Ligands which elute off this second column will be those ligands which bind at the site of interest which were displaced by the known ligand immobilized to the column. When the eluate passes through the immobilized column, weakly bound complexes may dissociate when the separation of target and ligand occurs very rapidly, maintaining the equilibrium will become dependent on the off-rate (k_2). This means that a low

exploiting the fact that, subsequent to complex dissociation, there is a low probability that the ligand will contact a target bearing surface again before elution from the system.

In various embodiments, additional dimensions relate to selecting and recovering ligands with a preselected affinity for a target of interest. Preselected affinity refers to the ability of the ligand to specifically bind to the target molecule, i.e. the strength of the interaction between target and ligand. Typical values for the preselected affinity are in the order of 10^{-3} l/mol to about 10^{-4}l/mol at a minimum, and are preferably about 10^{-8} to about 10^{-10} l/mole. The preselected affinity value is dependent on the environment in which the ligand and target molecule are found, as well as their concentration. In some applications, a lower affinity is acceptable, while in other applications, the affinity value may be much higher. One skilled in the art can routinely determine the desired affinity constant depending on the particular target and application of interest.

It is also possible to select specific binding conditions through the selection of the mobile phase with which the column is washed subsequent to peptide binding. To determine T for a particular application, one skilled in the art can calibrate an affinity column using any of the methods known in the art. For example, one may calibrate the column by introducing a pure sample of a ligand having a known affinity constant for binding to the immobilized target. The ligand is then loaded onto the column. Serial column volumes are then passed through the column, and directly introduced into the accumulator, until one obtains the ligand with the known affinity constant K'. T can then be calculated based upon the following equation:

$$T = \frac{K'}{K_{(known)}}$$

Thus, K', also known as the retention factor is defined by the equation

$$K' = K \times (T)$$

In practice, therefore, if one desired to obtain a ligand having an affinity constant K of 10^{10}, T could be set at, for example, 10^{-8}M. The ligand having the preselected affinity K will be obtained upon passing approximately 100 column volumes of solvent through the column.

of target. The concentration of the unbound ligands varies with the initial target concentration, and following automated data manipulation, the affinity constant can be determined.

The screening procedure can be used repeatedly to detect ligands or analytes from these sample libraries, having a preselected affinity. One may prefer the binding constant gradient obtained for one ligand to the target of interest to that of another, depending upon the desired elution conditions. For example, the strength of binding in a series of solutions containing methanol at increasing concentrations or solutions with increasing salt concentrations simulating elution gradients can be used. In this way, one can evaluate the comparative behavior of a number of ligands at a multitude of elution conditions.

The target immobilized column may be directly coupled to an accumulator, such as a reverse phase column. The method in various embodiments employs a tandem column chromatographic technique in which the target-ligand complex captured by the first column is dissociated and eluted directly onto an accumulator such as a reverse phase column for caption of the dissociated ligands. The bound complex can then be dissociated from the column, and introduced into the RP column. The accumulator should be suitable for selecting the bound ligand-target complexes eluted from the chromatography column. If the accumulator is, for example, a size exclusion chromatography column, one can assume that if the flow rate through the SEC column is rapid, there is little time for the dissociation of the target-ligand complex. At a slow flow rate, the opposite is true. As discussed in greater detail above, there is a direct correlation between the amount of washing of the complex on the first column with the relative affinities of the ligands.

The methods of the invention also encompass the preparation of pharmaceutically active compositions wherein a ligand in a sample having a preselected affinity K for a target molecule of interest is identified. The ligand is identified by loading a column with a known concentration T of target molecules; passing a sample through the column to bind ligands in the sample, and passing through the column a series of column volumes of solvent. A subset of the column volumes exiting the column are then passed to an accumulator to immobilize thereon ligands having the preselected affinity. The ligand is then eluted, and the ligand, or a derivative thereof is

C. THE INTERFACES

The system and apparatus of the invention may also include a coupling interface for capturing the eluent from one dimension and introducing it to one or more different dimensions. The interface may optionally contain a buffer system to effectively desalt, dilute or remove organic solvent from the eluent of one dimension prior to loading in the next dimension. Thus, in some situations, one may incorporate a buffer exchange in the interface. The buffer exchange may be a mixed bed matrix, packed with cation and anion exchange sorbent. Alternatively, the buffer exchange may comprise a separate column for each of the cation and anion exchange sorbent.

Tandem columns of cation exchanger and anion exchanger, or a mixed bed exchanger can be used to capture biomolecules from the eluent of a column. Thus, for example, if one eluted a desired ligand from any column in the system with an acid, the eluant could be directed into a tandem buffer exchange to alter the pH prior to introduction to the next column. The eluant may first be introduced into a cation exchange column which will capture the ligands from the eluant. The cation column is then washed with a neutral pH buffer, and the desired ligands captured onto the subsequent column, i.e. an anion exchange column. The ligands can then be eluted off this second column with a buffer or solvent optimized for introduction into the next dimension of the system.

The buffer exchange interface is particularly valuable in multi-dimensional systems where the elution buffer of, for example, an affinity column, is not suitable for introduction into a mass spectrometer. Thus, the desired ligands are washed from the affinity column, passed through a cation column and an anion column prior to introduction into the mass spectrometer.

In an alternative embodiment, the claimed invention relates to the tandem use of an affinity column, and a column having immobilized thereon an enzyme for digestion. Thus, a desired ligand may be captured in the first column, an affinity column, and eluted with acid. The ligand in the eluant may then be captured on a cation exchange column. The desired ligand can then be eluted off of the cation exchange column with a buffer optimized for the next column, i.e. a trypsin column.

sampling valve 22 is cycled at a predetermined sampling rate to insert a sampling volume into the liquid chromatography stream to take a sample of the liquid chromatography eluant, and then insert it into the mass spectrometer stream to analyze the sample.

More specifically, the liquid chromatography eluant 18 from the LC column 12 flows into an input port 20 of a sampling valve 22 and out through an output port determined by the selected position of the valve. An LC detector 26 can be connected to an output port 28 of the sampling valve 22 to accept the LC eluant from the LC column directed through the sample valve. Alternatively, the LC detector can be placed between the LC column and the input port 20 of sampling valve 22 with similar results.

Sampling valve 22 can, for example, be a rotary multi-port valve capable of two possible by-pass configurations and controlled by a sample controller 24. Figure 1 shows the sampling valve in a first position A, and Figure 2 shows the sampling valve in a second position B. In valve position A the LC stream sampling position, the eluant 18 from LC column 12 enters the sampling valve through input port 20 and exits the valve through output port 30. Output port 30 is connected to another input port 34 by a length of tubing 32 defining a sample loop having a predetermined sample volume. In position A, LC eluant flow through the sample volume into input port 34 and is directed to output port 28 for detection.

A MS precision pump 36 pumps MS analysis stream 38 into another sampling valve input port 40, and MS 44 accepts its analysis stream from another sampling valve output port 42. In valve position A, the MS stream 38 goes through input port 40 directly to output port 42 and to MS 44.

Upon switching the sampling valve 22 from position A to position B (Fig. 2), the MS infection position, the predetermined sample volume of LC eluant is trapped in the tubing 32, and transferred into the MS analysis stream 38. Specifically, in position B, sample valve 22 directs the LC eluant through input port 20 directly to output port 28 and to the LC detector 26. However, the MS analysis stream 38 is now directed through sample loop tubing 32 into MS 44, thereby injecting the trapped sample volume of LC eluant in tubing 32 into the MS analysis stream.

of the invention may have a software interface, i.e. controller, which allows for a wide variety of assay formats, and may optionally include a spectrophotometric detector. The software interface may be tailored to a broad range of specifications and comprises three functional areas: instrumental control, methods development, and analysis. The instrument control may provide a graphical interface to each physical element of the system, from buffer selection to sample preparation, through to detection and fraction collection. The status of the system may be continuously monitored and displayed on the computer screen.

D. DETECTION

Any method of detection known in the art is suitable for use in the claimed invention. Thus, ligands, or target molecules may be labelled to render them detectable. Thus, either the target or the ligand may be labelled with a detectable moiety such as enzymes, fluorophores, chromophores, radioisotopes, electrochemical moieties and chemoluminescent moieties. Additionally, the invention contemplates a composition comprising a first binding partner having a detectable moiety which is intrinsic, e.g. a functional group capable of detection.

Additional methods of detection include, for example, any apparatus for obtaining mass-to-charge ratio, including, but not limited to: matrix-assisted laser desorption ionization/plasma desorption ionization, electrospray ionization, thermospray ionization, and fast atom bombardment ionization. Additionally, any mode of mass analysis is suitable for use with the instant invention, including but not limited to: time of flight, quadrapole, ion trap, and sector analysis. The preferred method of detection and analysis is an improved time of flight instrument which allows independent control of potential on sample and extraction elements, as described in copending USSN 08/446,544 (Atty. Docket No. SYP-111, filed May 19, 1995).

The methods development component allows the user to create automated assay methods, including setup of injection sequences and sample preparation. Dilution and derivatization may be included in sample preparation.

Assay analysis allows the quantification of individual runs, and may incorporate parameters such as standard curves or dose levels to be prepared. Thus, a standard curve may be developed from a set of assays by automatically measuring the detector response and fitting this

The kits may optionally be configured to detect ligands as well as to analyze, and obtain said ligands, and may include, for example, an interface for rendering a sample solution compatible with additional analyses. The kits of the invention may be used to identify and obtain ligands in samples. The ligands, or derivatives or modifications thereof can be used for a variety of purposes, such as lead compounds for drug discovery. Optionally, one may use the ligands or modifications or derivatives thereof to prepare a pharmaceutically active composition.

Pharmaceutically active compositions of the invention are prepared by identifying ligands in samples having a preselected affinity K for a target molecule of interest. The ligand may be identified by loading a column with a known concentration T of target molecules, passing a sample through the column to bind ligands in the sample thereto, and then passing a series of column volumes of solvent through the column. A subset of the column volumes exiting the column can be introduced to an accumulator, to immobilize thereon ligands having the preselected affinity, and eluting the ligand.

Compositions of the invention may optionally include adjuvants.

Advantages of the present invention include speed, reproducibility and automation. It is preferable to use particles capable of perfusion chromatography since perfusion particles allow the system to operate at very high flow rates while maintaining both high sample loading capacity and chromatographic resolution.

As will be understood from the above description and the examples given below, the claimed invention is amenable to numerous configurations which may be chosen on the basis of the desired application or the sample. To further exemplify the invention, several configurations are enumerated in more detail below. In certain embodiments, as depicted in Figure 3, the apparatus of the invention 110 comprises a sample input 112 for introducing sample into the apparatus 110 into a first column 114 to partition, based on a first physico-chemical property, candidate ligands, or complexes thereof with a target molecule, to generate a first exit stream 116. First exit stream 116 is optionally directed to a second column 118 to partition candidate ligands based on a second, different physico-chemical property, to generate a second exit stream 120.

removing organic solvent from the exit stream 116 prior to reintroduction to the system through valve 124. Exit stream 152 is then introduced into third column 132. Third exit stream 134 can then be introduced to a splitter 140, and a sample directed to the mass spectrometer 136, and ultimately, the information is transmitted to an output display 138.

EXAMPLES

Example 1: Screening of a Synthetic Peptide Combinatorial Library (SPCL) using an antibody against β-endorphin as a target

Example 1A: Preparation of the Target Immobilized Affinity Column and the Control Column

Monoclonal antibody (mAb) chosen (mouse IgG2a, clone 3E-7, Boehringer Mannheim, Indianapolis, IN) was raised against human β-endorphin and recognizes the amino terminus of β-endorphin, YGGFL. The purchased mAb (280mg resuspended in 1ml H_2O) was passed over an XL cartridge (2.1 x 30mm) consisting of protein-G coupled to POROS® perfusion chromatographic media (PerSeptive Biosystems, Framingham, MA) by making 10 x 100ml injections on a BioCAD™ 20 Workstation (PerSeptive Biosystems, Framingham, MA). Protein-G binds to the Fc region of antibodies with high affinity. The mAb was subsequently cross-linked to the protein G using the standard methods and materials provided with the XL column. In brief, this consisted of passing 14 ml of cross-linking solution (100 mM triethanolamine, pH 8.5, 7.8 mg/ml dimethyl pimelimidate (DMP)) over the column at a flow rate of 0.5 ml/min. The cross-linking reagent was quenched by subsequent injection of 2 ml of 100 mM monoethanolamine, pH 9.0. The sensor cartridge was washed with PBS, pH 7.4 at 0.5 ml/min for 2 min followed by a further injection of 2 ml of quench solution and washed as above. The antibody was efficiently immobilized to the column as demonstrated by the lack of reactivity to coomassie stain upon SDS-PAGE of the flow through. A second XL column (without antibody) was treated with cross-linking reagents and washed as for the affinity column for use as a "control column".

Example 1B: Control Screening of the Immobilized Target with the Known Epitope

The target immobilized affinity column prepared above was plumbed in tandem mode to a vydac reversed phase C-18 column on a BioCAD™ Workstation using the configuration shown in Figure 5. This configuration allowed independent equilibration and washing of the affinity and

7.4. Peptides affinity captured by the antibody was eluted from the "target column" directly onto the C-18 column for resolution. The amount of the peptide recovered, as calculated from the peak area, was approximately 1.2 nmoles. The theoretical capacity based on the quantity of mAb loaded onto the affinity column was 3.8 nmol demonstrating that approximately 25% of the binding sites of the mAb are available in a conformationally active form.

Example 1E: Determining the Capacity of the Target Immobilized Affinity Column

The capacity of the column was examined by injecting increasing larger quantities of the peptide (YGGFL) utilizing the loading template of the BioCAD™ Workstation. The amount of the bound peptide (as calculated from the peak height) reached saturation at about 1.2 nmoles. Interestingly, the amount of peptide bound to the antibody was independent of the flow used to inject the peptide. Increasing the flow rate from 0.2ml/min to 5 ml/min did not affect the recovery. This result suggests a rapid interaction of the peptide with the antibody during the loading process when a perfusive packing material and elution conditions are used. The EC_{50} value (50% of the saturating amount) for the peptide is approximately 30 nmols. This value correlates well with the affinity constants determined previously for binding of YGGFL peptide to 3E-7 by competitive radiolabelled binding assays (See Lam *et. al.*, *Biorganic Med. Chem. Letts.* 3:419-424 (1993)).

Example 1F: Screening of the SPCL using the Target Immobilized Affinity Column

The diversity of the library was first assessed by running a small aliquot on the vydac C-18 column under the same conditions used for elution of bound material from the XL column. The large number of peaks exhibiting significant absorbance at the indicated wavelength are suggestive of the diversity of the library.

Using the conditions established for purified YGGFL binding to the mAb column, the XXXFL library was screened for moieties recognized by the target immobilized mAb affinity column. The library (containing 2.8nmols of each peptide) was loaded onto the "target column" , the unbound material was washed with 10 Column volumes of PBS, pH 7.4. Finally, the affinity bound material was eluted directly onto the vydac C-18 column with 12 mM HCl. Elution of the C-18 column (with a 4-80% ACN gradient as described above) revealed approximately 10-12 resolvable peaks. The elution profile shows that, one of the peaks observed exhibits a retention

expected to interact with this column due to the presence of two charged phosphate groups at the lipid A region of the molecule. Hence cationic molecules capable of binding to LPS would only be retained on column 1 if they were bound to this molecule. Material retained on column 1 was eluted directly onto column 2 where it was captured. Elution of column 2 allowed the resolution of peaks arising from column 1.

Example 2B: Synthesis of the XXXFL Library

The library XXXFL was made on Fmoc-Leu WANG resin using standard procedures in an Advanced Chemtech librarian peptide synthesizer. Prior to screening the library (consisting of approximately 5800 pentamers) it was divided into an anionic and cationic fraction based on retention on the weak anion exchange column (material binding to the column when injected in 50 mM tris, pH 6.7 was designated as the anionic fraction, while that passing through was the cationic fraction). Only the cationic fraction was screened using this paradigm. However, the cationic fraction represented > 2/3 of the total library based on peak area for the two fractions.

Example 2C: Screening of the XXXFL Library

Screening the library for binding to LPS was performed as follows. The cationic fraction of the XXXFL library was incubated (30 min/RT) with 1-3 mg/ml of LPS (serotype O55;B5; Sigma Chemical Co., St. Louis, MO) in 50 mM Tris, pH 6.7. At this time the incubation mixture was injected and run over column 1 on the BioCAD™ Workstation (equilibrated in the same buffer). The column was run at 4 ml/min. After washing with the appropriate number of column volumes of equilibration buffer (1 CV = 1.66 ml), the column was purged and eluted (using 8 mM HCl, 1M NaCl) directly onto column 2 by switching the latter column in line with column 1 during the elution process. Material captured on column 2 was eluted using a gradient from 12 mM HCl in water to 80% ACN, 6 mM HCl. Peaks were collected, further purified by rerunning on a vydac C-18 column (4.6 x 250 mm), and analyzed by Mass Spectrometry (on a Voyager™ MALDI-TOF instrument; PerSeptive Biosystems, Framingham, MA) and peptide sequencing (Hewlett Packard). Peptides identified were synthesized and binding of these molecules to the

A BioCAD™ Workstation was plumbed in the tandem column configuration. Column one was an immobilized rHsp70 affinity column (2.1 X 30 mm) and column two was a POROS® R2 reversed-phase column (2.1 X 100 mm). The affinity column (column one) was washed with screening buffer before the library (natural protein digest library [PDL], 100 µg) was injected onto the affinity column at a flow rate of 0.2 ml/min. The affinity column was then washed with screening buffer in increasing numbers (5, 10, 20, & 40) of column volumes (CVs) at 0.2 ml/min. (1CV = 100 µL) The POROS® R2 column was then switched in-line downstream of the affinity column. Bound material was eluted off the affinity column with acid directly onto the in-line RP column. The affinity column was then taken off-line and washed back into screening buffer. Finally, the POROS® R2 column was eluted with an ascending acetonitrile gradient (0-80%) in TFA (0.1%).

Figure 6 shows the RP column portion of the experiment, i.e., elution onto an in-line RP column after selection by a rHsp70 affinity column from PDL after different number of wash volumes. At 40 CVs only a single peak is selected ("**" representing a high affinity binder) suggesting that the protein represented by that peak has a high affinity for rHsp70. At lower wash volumes, e.g. 5 CVs, other peaks ("*" representing a low affinity binder) are seen. These peaks are absent at 10 and higher CV washes, suggesting that they are low affinity binders.

Example 2E. Subtractive Screening-Selection of Target Specific Binders

A multi-target column format can be used to screen ligands for their ability to bind to a certain target and for their inability to bind to a second target in a single process. This technique should be generally applicable for selecting binders that differentiate between two different targets. For example binders can be selected that differentiate between wild type and mutant protein. For another example, ligands can be selected that bind to a pathogenic target but not to the homologous host target. Subtraction can be done in one chromatographic process or in parallel chromatographic runs with subtraction done at the analysis level.

A library has been screened using a SEC subtractive screening protocol with human Hsp70 as the host protein and its E. coli counterpart, DnaK as the pathogen target. A BioCAD™ Workstation was plumbed in the tandem column configuration. Column one was a size exclusion column (SEC) and column two a POROS® R2 reversed-phase (RP) column (2.1 X 100mm). Protein (50µg) was pre-incubated with library (100µg) and samples (100µL) were injected onto the SEC at a flow rate of 1 ml/min. The protein peak was either collected for mass spec. analysis

column was then eluted with an ascending acetonitrile gradient (0-80%) in TFA (0.1%). Finally, the SEC was equilibrated back into screening buffer.

Total peptides eluted from rHsp70 and from DnaK were analyzed by MALDI-TOF. Figures 7A, 7B, 7C and 7D show the MALDI-TOF spectra for such experiments. A number of peaks are present in the rHsp70 sample incubated with the PDL (Fig. 7A) that are not seen in the control incubations with rHsp70 (Fig. 7B) or PDL (Fig. 7C) alone. This indicates that these peptides are binding to rHsp70 itself or that there is insufficient separation of bound and unbound library. DnaK incubated with PDL (Fig. 7D) also binds peptides originating from this library. DnaK binds a different ensemble of peptides, although some are in common with rHsp70.

Example 2F. Bimolecular Screening-Selection of Site-Specific Binders

Tandem columns consisting of an affinity column plumbed in-line to a RP column can be used to screen libraries for ligands that bind to a specific site on the target molecule via comparison of the eluants from a column containing both target molecule and a known ligand with the eluants from a column containing only target molecule.

The lectin Concanavalin A (Con A) has been used. Ligands were screened for specific binding to the sugar site of Con A as an example of the bimolecular approach to screening a mixture of components for interaction with a target. Biotinylated succinyl Con A was immobilized onto a streptavidin POROS® support (BA cartridge, 2.1 X 30 mm). A library consisting of the sequence XXXXX (where X represents any of 20 natural amino acids excluding cysteine) was passed over the affinity column, and material interacting with this support was subsequently eluted and captured on a RP column. In order to identify ligands that had specificity for the sugar-binding site, the peptide library was passed over the test column in the presence and absence of a ligand for Con A (methyl-α-D-mannopyranoside, 33 mg/ml). After exposing the column to 10 CVs of CAB buffer (0.2 ml/min.), the remaining peptides were acid eluted onto a RP column. These peptides were eluted from a RP HPLC column using an acetonitrile gradient and the fractions were collected, pooled and sequenced. Recovery of each amino acid was expressed as a percentage of the total amount of amino acids recovered at each cycle of sequencing (AA%). Amino acid enrichment was then expressed for each amino acid as follows:

conditions. The peptide(s) selected by either one of the above screening methods were characterized by mass spectrometry (MALDI-TOF) and Edman sequencing methods. The peptide(s) were then immobilized on POROS® media and evaluated for specificity, selectivity and capacity to bind IgG from serum. Various coupling chemistries (direct synthesis or off-line immobilization), the effect of ligand density, the activation chemistry, and the nature of interaction with IgG were investigated for some of the selected peptide(s).

Example 3: Screening

All proteins and reagents for buffers were obtained from Sigma Chemical Co (St. Louis, MO) unless otherwise specified. Anti-IgG (Fc specific) antibodies were purchased from Biodesign International (Kennebunk, ME). Screening by SEC and RP Chromatography was performed on the INTEGRAL™ chromatography Workstation (PerSeptive Biosystems, Inc., Framingham, MA). Screening by immobilized target (IgG) column in tandem with RP column was performed on the BioCAD™ 20 Workstation (PerSeptive Biosystems, Inc., Framingham, MA). Size exclusion column (Superdex 200 HR 10/30, molecular exclusion limit 150,000 daltons to 6000 daltons) was obtained from Supelco (Bellefont, PA). Vydac RP C_{18} column (4.6 mD x mmL) was obtained from Separation Science, Hesperia, CA. POROS® Self Packing device, POROS®-Protein A, POROS® HQ and POROS® CM columns were obtained from PerSeptive Biosystems (Framingham, MA). Mass spectral analysis was performed on the Voyager™ BioSpectrometry Workstation with linear analyzer and a 337nm Nitrogen laser, from PerSeptive Biosystems, Inc./Vestec Mass Spectrometry Products, Framingham, MA. Peptide sequencing by Edman degradation was done on the Hewlett Packard Series II 1090 liquid Chromatograph. SDS gel electrophoresis kit was obtained from Novex Biochemicals (San Diego, CA).

3B: *Diversity of the peptide library*:

To determine the diversity of each the peptide libraries, the sequences of all the proteins used to generate a library were obtained via the Entrez program and retrieved into the GPMAW program. The GPMAW program simulates the enzymatic digestions and generates information on the number, sequences and masses of all the possible peptides generated by such three batch digestion. This information was very valuable in predicting the extent of diversity and confirming the source, mass and sequence of the peptide(s) obtained after the screening.

3C: *Immobilization of IgG(s)*:

1 mg POROS® EP (epoxy) was suspended in 5 ml 0.1 M phosphate buffer (pH 9) containing 20 mg IgG. After the beads were well suspended, 4 ml 0.1 M phosphate buffer (pH 9) containing 2 M NA_2SO_4 were added to the mixture and it was shaken overnight at room temperature. The beads were then washed with 10 mM PBS (pH 7.5) and stored in the refrigerator before they were packed into columns.

3D: *Peptide Screening Protocol*:

1) Solution phase Peptide(s) Screening through Size Exclusion - Reversed Phase columns: 5 mg each of either mouse IgG (whole) or the Fc fragment was incubated with 20 mg of natural library and protein A and G digests, respectively. The mixture was dissolved in 1 ml of a mixture of 25 mM sodium phosphate buffer and 0.15 M NaCl (pH 7) overnight at 4°C. After incubation, several chromatographic runs through the Superdex SEC and RP columns were conducted with the protein and peptide mixture injected through the Superdex column during each run. The flow rate for the Superdex column was 0.75 ml/min with the 25 mM sodium phosphate buffer and 0.15 M NaCl (pH 7) mixture. The early eluting protein peak was collected directly onto the RP column. The remaining portion of the peak was washed off the SEC. The mouse IgG (whole) or Fc fragment and its associated peptides were then eluted from the RP column under the following conditions:

Flow rate:	1 ml/min
solvent A:	0.1% TFA/DIW
solvent B:	0.1% TFA/85% ACN/15% DIW
Gradient conditions:	0-100% B for 30 CVs.
	01.5 ml fractions were collected and lyophilized.

1) Immobilization of peptides via N-terminal end: 1g POROS® AL (aldehyde) was suspended in 10mM PBS (pH 7.5) containing 100 mg sodium cyanoborohydride ($NaCNBH_3$). 5 to 20 mg of each of the peptides selected by various screening procedures was added to the resin and the mixture was shaken overnight at room temperature. 100 mg sodium borohydride ($NaBH_4$) was then added and the mixture was shaken for another 2hrs. The beads were then washed with PBS and packed into 4.6 mm D x 100 mm L column using POROS® Self Pack® device.

2) Direct synthesis of peptides on POROS®: The 19-mer peptide (TVTEKVIDASELTPAVT) selected from protein A and G digests was directly synthesized on 20 μm amine-functionalized particles by standard FMOC chemistry. About 700 mg of resin was dry packed into a 4.6 mm D x 50 mm L PEEK column equipped with 2 μm frits. The packed column was attached with appropriate adapters to a PerSeptive Biosystems 9050 plus continuous flow peptide synthesizer. Upon completion of synthesis, the resin was dried and deprotected using 95% TFA/5% triisopropylsilane for 24hrs. The final peptide-support conjugate was packed using POROS® Self Pack® column packing device at flow rate 10 ml/min onto 4.6 mm D x 50 mm L column for evaluation as affinity supports.

3G: *Analytical Methods*:

1) Purification of IgG (whole) from human serum on peptide column: 100 μl of 1:10 diluted serum was injected onto the peptide column which was equilibrated with 20 mM tris (pH 8.0) at 5 ml/min. The bound proteins were eluted with 0-1 M NaCl gradient in 15 CVs. Fractions were collected manually and concentrated by Speed Vac for subsequent analysis by SDS-polyacrylamide gel electrophoresis.

2) Purification of IgG (whole) from human serum on POROS®-Protein A column: 100 μl of 1:10 diluted serum was injected in the protein A column, which was equilibrated with 10 mM phosphate buffer containing 0.15M NaCl (pH 7.5) at a 5 ml/min flow rate. After injection and 5 CVs wash, the bound portion was eluted in a single step with 10 mM HCl. The bound fractions were collected and analyzed by SDS-Polyacrylamide gel electrophoresis.

3) Purification of IgG (whole and Fc fragment) on POROS®-peptide (TVTEKPEVIDASELPAVT) column: The 19-mer peptide column (4.6 mm D x 50 mm L) was

Natural Peptide Library:

Both solution phase and solid phase peptide(s) screening of mouse IgG (whole fragment) with the natural peptide library yielded one peptide of mass 1633 daltons and amino acid sequence CAQCHTVEK. Database search revealed that this peptide is a tryptic digest of cytochrome c (one of the proteins in the library) with a heme group covalently attached to the two cysteines at amino acid positions 14 and 17 of the protein.

The CAQCHTVEK peptide with heme group was immobilized on POROS® AL (aldehyde) via the N-terminal end. This POROS®-peptide conjugate was used to separate IgG from serum at pH 8 under a 0-1M NaCl gradient. At pH 8, IgG was purified with comparable purity to that of IgG separated on POROS®-Protein A column. The capacity of the POROS®-peptide column was determined to be 10 mg/ml column volume which is comparable to the binding capacity of POROS®-Protein A column. To determine the nature of interaction, the purification profile of IgG separated on the POROS®-peptide column was compared with the IgG purified on standard ion exchange columns such as POROS®-CM and POROS® HQ. Results indicated that, under similar conditions, peptide columns exhibit predominantly ion exchange characteristics with secondary hydrophobic interactions and have a higher selectivity for IgG from serum than either of the ion exchange columns. The effect of the varying loading densities of peptide (from 10 mg/g POROS® to 100 mg/g POROS®) on specificity and capacity for IgG binding was also investigated. The specificity of the peptide for IgG binding was also investigated. The specificity of the peptide for IgG did not vary with varying ligand densities, but the nature of interaction of IgG varied. At low loading density (10 mg/g POROS®) IgG bound primarily via ionic interaction requiring elution of bound IgG with salt gradient. At higher loading density (100 mg/mg) IgG bound strongly and eluted with acid buffer. The binding capacity varied from 1-2 mg/ml column volume at lower ligand density to 30 mg/ml column volume at higher ligand densities. The heme peptide POROS® bound very weakly to HSA and only under very hydrophobic conditions (200 mM sodium sulphate, pH 7 buffer).

When the heme peptide was immobilized via the carboxyl groups of heme and the free C-terminal end, no IgG binding was seen indicating that free carboxyl groups of the peptide were very important for binding to IgG.

A simplified analog of the heme peptide (GAQGHTVEK) was synthesized and immobilized on POROS® AL via the N-terminal end. At pH 8, this GAQGHTVEK-POROS®

two of the peptides namely, TVTEKEPEVIDASELTPAVT and TVTEKPEV are part of the Fc binding domain of recombinant protein G.

Polyclonal Antibody digests:

Polyclonal antibodies are an interesting and logical source of peptides since they have specific antigen binding sites. A synthetic antibody fragment against lysozyme has been used as a ligand in immunoaffinity chromatography. This fragment was generated by molecular modeling of lysozyme and its antibody (Welling, G.W. *et al.*, (1990) *J. Chrom., 512*:337-343). Single chain antibodies that bind with weak affinities have also been generated against many targets by phage display (Griffiths, A.D. *et al.*, (1994) *The EMBO J., 13*(14):3245-3260). To date there has been no report of selection of target specific peptide(s) isolated from polyclonal antibody digests. Tryoptic digests of denatured anti-IgG (Fc specific) polyclonal antibodies raised in rabbit, goat and sheep were run through a POROS® epoxy column immobilized with IgG. The bound peptides were eluted onto an RP column and characterized. The amino acid sequence was determined to be GAQGHTVEK. A database search revealed that the GAQGHTVEK sequence is a part of the variable region of the light chain of IgG. Note that the HTVEK motif is also found in the heme peptide of cytochrome c. The heme peptide has been shown, as above, to bind IgG. Additionally, the TVEK motif is similar to the TVTEK sequence found in the IgM heavy chain, T-cell receptor (beta chain) and also IgG binding proteins such as protein G and protein LG. Protein LG, a hybrid molecule of protein L and G, binds to intact IgGs, as well as Fc and Fab fragments and IgG light chains. The characteristics of GAQGHTVEK peptide as affinity surface for IgG binding have been discussed above. The most important and novel feature is that from a mixture of denatured antibodies, one peptide was isolated that was selective for IgG.

By choosing different libraries, peptides have been chosen that selectively bind to different portions of IgG (either the Fab or the Fc fragment). There is remarkable similarity in the sequences of some of the IgG binding peptide(s) isolated from different protein sources. None of these peptides were shown to bind IgG previously. The specificity of the peptide(s) for IgGs from various species varies depending on the orientation, activation chemistry and the density of immobilized ligand. Finally, this invention provides proof of the concept that both chromatographic peptide(s) screening technologies (solid phase and solution phase) are comparable and yield credible results.

d) a chelation surface;

e) a polysaccharide surface; or

f) a polynucleotide surface.

5. The method of claim 1 wherein at least one of said first and second physico-chemical properties is selected from the group consisting of:

a) the binding constant K_b of a candidate ligand for a target molecule;

b) the on rate component of the binding constant of a candidate ligand for a target molecule; and

c) the off rate component of the binding constant of a candidate ligand for a target molecule.

6. The method of claim 1 comprising the step of conditioning said solvent in said exit stream prior to partioning in said second column.

7. The method of claim 1 wherein the first column comprises an affinity column comprising immobilized first target molecules.

8. The method of claim 7 wherein only candidate ligands which fail to bind to said first target molecules are passed through the second column thereby to eliminate candidate ligands which bind to the first target molecule from subsequent screening.

9. The method of claim 8 wherein candidate ligands which bind to said first target molecules are desorbed from said first column and passed through the second column thereby to eliminate candidate ligands which fail to bind to the first target molecule from subsequent screening.

10. The method of claim 8 wherein said second column comprises an affinity column comprising immobilized second target molecules.

11. The method of Claim 9 wherein said second column comprises an affinity column comprising immobilized second target molecules.

12. The method of claim 1 wherein said first column comprises a size exclusion column and, prior to step A, said library is mixed with a target molecule to form candidate ligand/target molecule complexes.

21. A method of detecting the presence of a ligand having a preselected affinity K for a preselected target molecule in a sample of heterogeneous ligands dissolved in a solvent, the method comprising:

a) loading a column with a known concentration T of target molecules;

b) passing a sample through said column to bind ligands in the sample thereto;

c) thereafter passing through the column a series (n) of column volumes of solvent, wherein n is a number of column volumes between 1 and 10,000;

d) passing a subset KT of the column volumes exiting the column of step c) through a ligand accumulator to immobilize thereon ligands having said preselected affinity K wherein the subset of column volumes kp~KT, and,

e) eluting from the accumulator of step d) said ligands having said affinity K.

22. The method of claim 21 wherein, during step b), the velocity of the sample passing through the column is selected to modulate the on-rate component of the affinity constant of the ligand sought to be detected, wherein increasing the velocity of the sample through the column results in the binding in step b) and elution in step e) of ligands having a larger on-rate.

23. The method of claim 21 wherein, during step c), the velocity of solvent passing through the column is selected to modulate the off-rate component of the affinity constant of the ligand sought to be detected, wherein increasing the velocity of the solvent through the column results in the elution in step e) of ligands having a higher off-rate.

24. The method of claim 21 comprising the step of passing the eluate of step (d) through an interface to a mass spectrometer.

25. The method of claim 21 further comprising the step of independently optimizing the flow rate through the columns of step a) and b), and independently optimizing the flow rate of the eluate to a mass spectrometer for determination of the mass-to-change ratio of a ligand in said eluate.

26. A method for separating mixed ligand species dissolved in a solvent into separate fractions of ligands, each fraction being characterized by a different affinity or range of affinities for a preselected target molecule, the method comprising:

c) thereafter passing through the column a series of column volumes of solvent defining said solvent conditions;

d) passing a subset kp of the column volumes exiting the column of step c) through a ligand accumulator to immobilize thereon ligands having said desired high affinity, and

e) eluting said ligands from the accumulator of step d).

30. The method of claim 29 wherein a ligand eluted in step e) is characterized by a said high affinity K for said target molecule equal approximately to kp/T where T is the concentration of target molecules in said column.

31. A method of detecting, in a heterogeneous sample comprising multiple ligand species at least some of which bind a preselected target molecule with an affinity of at least about 10^4 M^{-1}, the presence of a ligand having a high on-rate, Ko, when said ligand and said target molecule are present together in preselected solvent conditions, the method comprising:

a) immobilizing a target molecule onto a column;

b) providing said heterogeneous sample in a solvent defining said preselected solvent conditions;

c) passing the sample of step b) through said column at a high linear fluid velocity so as to minimize residence time of ligands of said sample in said column thereby to bind selectively high on-rate ligands to said target molecules in preference to other ligands in the sample;

d) thereafter eluting said column to produce an output and identifying said high on-rate ligands.

32. The method of claim 31 comprising the additional step of passing the output of step d) through a ligand accumulator and eluting the accumulator to produce an output rich in said high on-rate ligand.

33. A method for the selective screening of a library of heterogeneous ligands to detect a desired ligand characterized by at least two different preselected binding characteristics to first and second target molecules, said method comprising the steps of:

a) combining a solution of heterogeneous ligands with a first target molecule under conditions such that candidate ligands bind to said first target molecule thereby to form candidate ligand/first target molecule complex;

39. The apparatus of claim 38 wherein said at least one additional column is interposed between said first and second columns and the output from said first column passes through said additional column before entering said second column.

40. The apparatus of claim 38 wherein said at least one additional column is disposed after said second column and is adapted to receive at least a portion of an output from said second column.

41. The apparatus of claim 37 further comprising a ligand accumulator connected through a valve to an output of said second column or a said additional column for capturing a subset of ligands characterized by said affinity for and preferential binding to said preselected target molecule.

42. The apparatus of claim 41 wherein said accumulator is a reverse phase chromatography column, which permits chromatographic separation of accumulated ligands.

43. The apparatus of claim 37 further comprising an instrument for determining a physico-chemical structure aspect of a selected ligand exiting a said column or accumulator.

44. The apparatus of claim 43 wherein said means is a mass spectrometer.

45. The apparatus of claim 37 disposed between consecutive columns for conditioning the solvent characteristics of an output stream of an upstream column for partitioning within a downstream column.

46. The apparatus of claim 37 for isolating selected ligands which will bind to target molecules from a first biological source but not to structurally related target molecules from a second biological source, wherein said first unit is an affinity column comprising immobilized target molecules from said second source, said second unit is an affinity column comprising immobilized target molecules from said first source, and said valve directs ligands which do not bind to said first for capture in said second unit.

47. An integrated multi-dimensional system for isolating and for obtaining physico-chemical data characteristic of ligands having an affinity for a preselected target molecule, said system comprising,

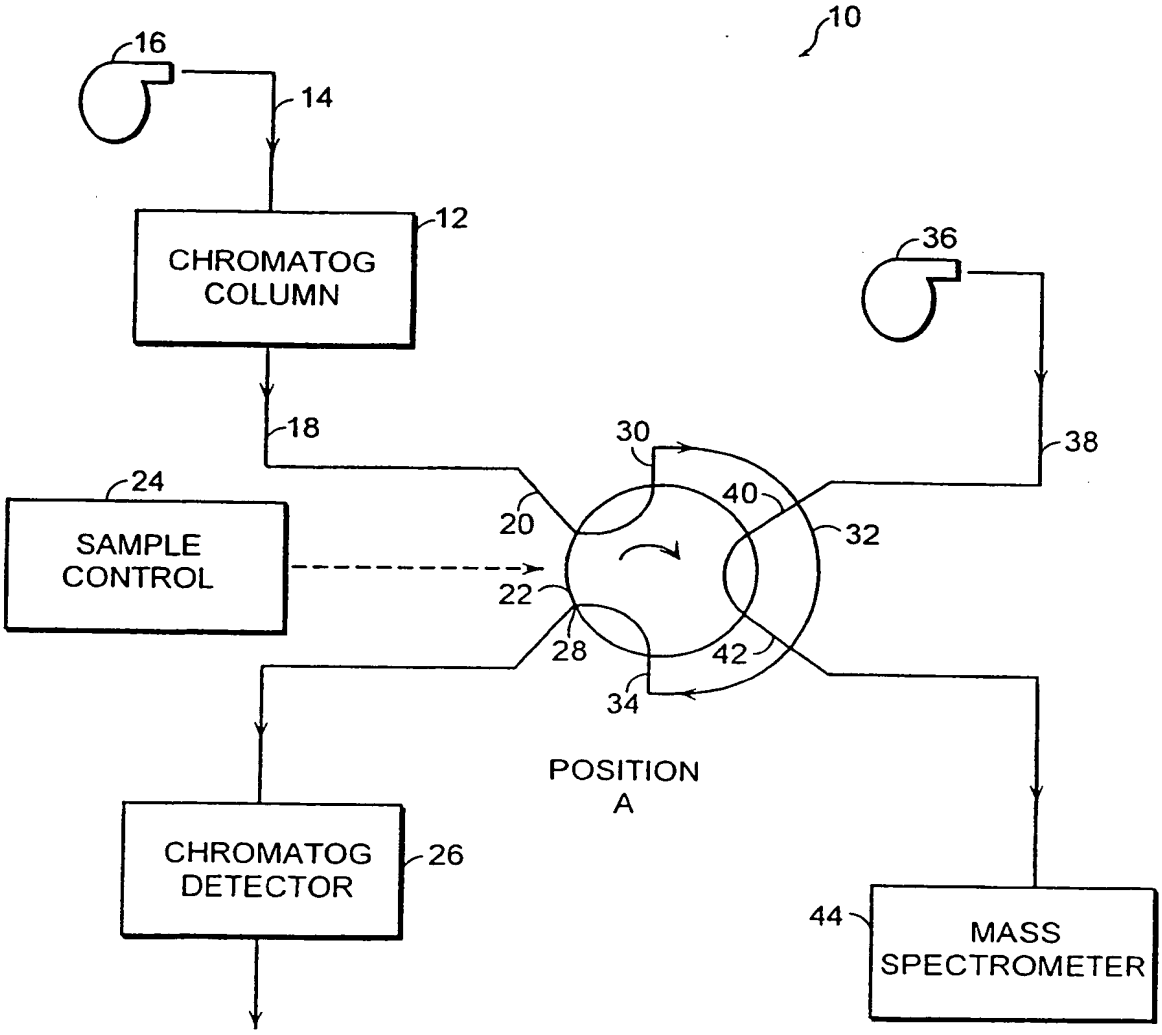


FIG. 1

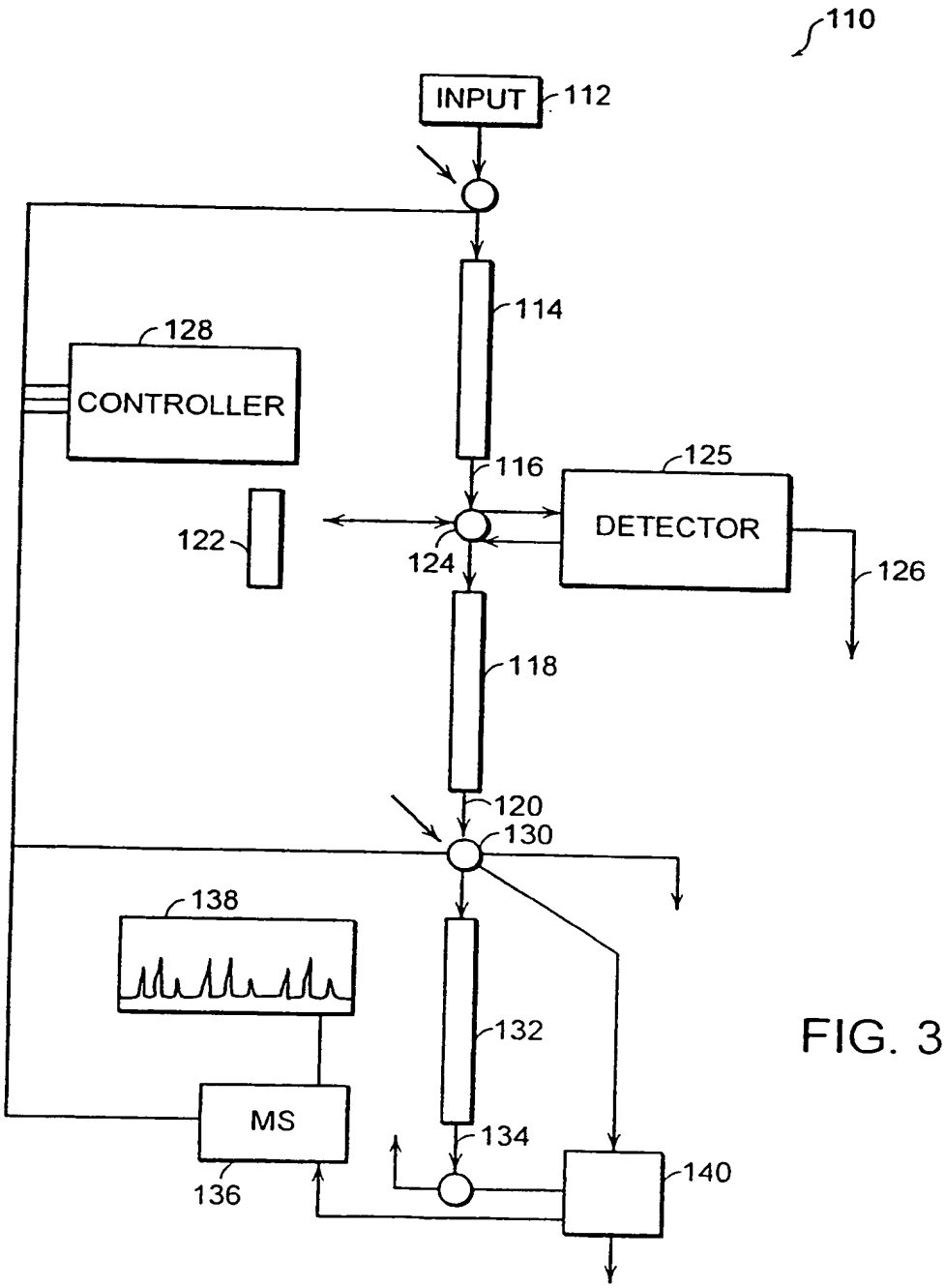


FIG. 3

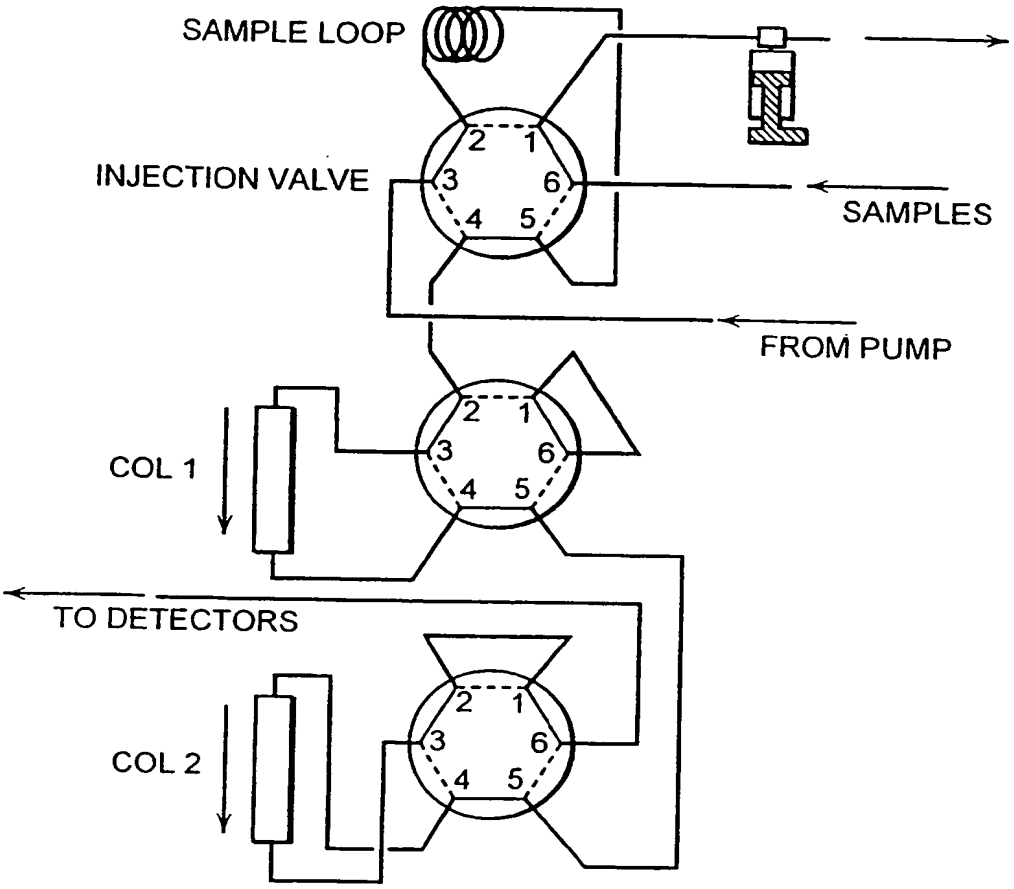


FIG. 5

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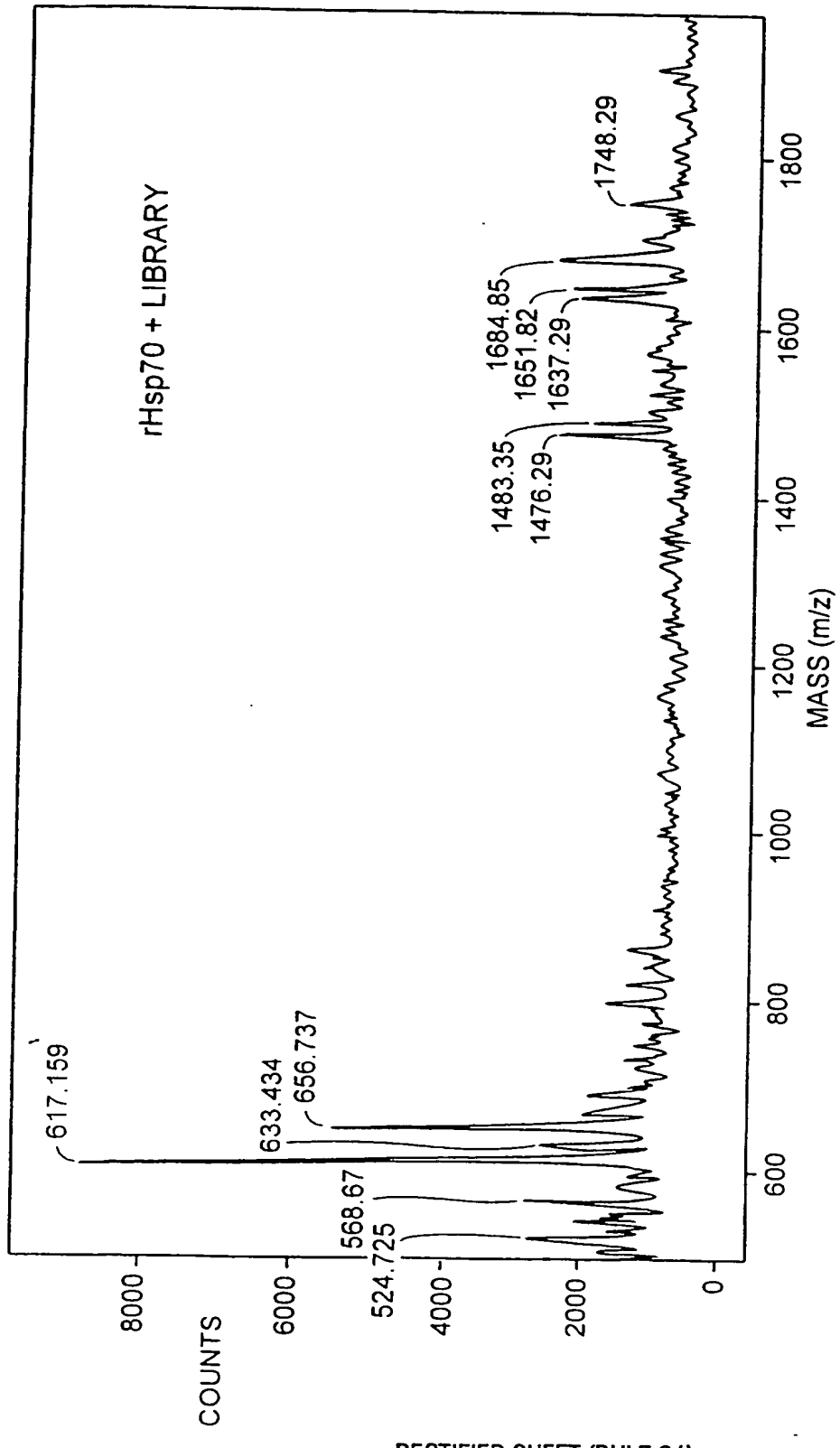

FIG. 7A

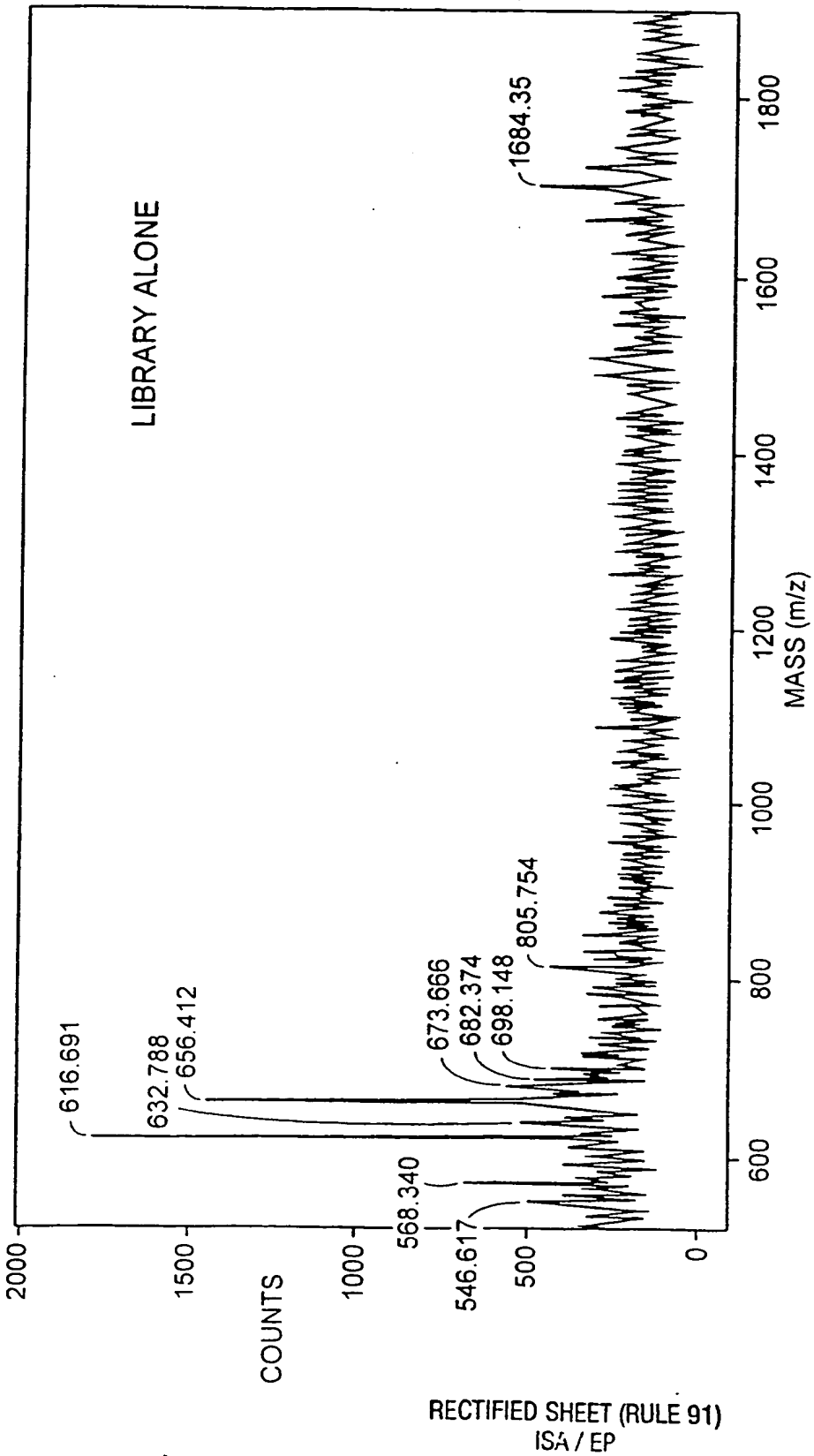

FIG. 7C

IDENTIFICATION OF PEPTIDE SEQUENCES
BINDING TO CONCANAVALIN A IN A
SUGAR-SPECIFIC MANNER*

RANKING AMINO ACIDS FROM GREATEST TO LEAST ENRICHMENT OVER CONTROL

NO INCUBATION ON-COLUMN						30MIN INCUBATION ON-COLUMN							
CYCLE #						CYCLE #							
1	H	R	Q	N		1	W	H	Q	R	I	V	N
2	H	N	Q			2	W	V	T	Q	I	P	
3	R	H/D/Q/N				3	W	V	T	Q	D	E	
4	S	R	W	Y		4	W	S	Q	V	E	N	
5	Y	R	Q			5	V	T	W	Q	E/D/G	N	R

*TABLE SHOWING ENRICHMENT OF AMINO ACIDS AT EACH CYCLE IN THE ABSENCE AND PRESENSE OF SUGAR

FIG. 8

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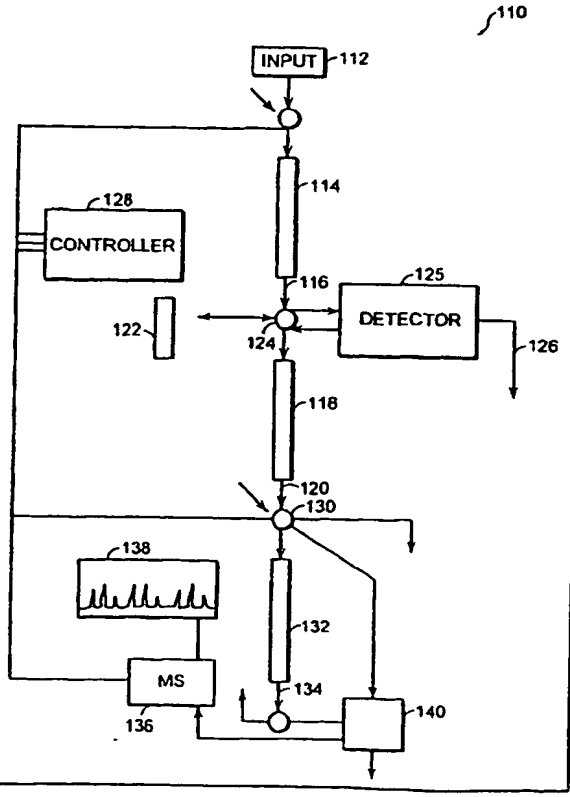
(57) Abstract

The invention provides novel methods for screening a sample to select a ligand to a target of interest and for obtaining information about the ligand and its binding characteristics. Specifically, the claimed multi-dimensional methods involve combining a solution of heterogeneous ligands with the target of interest to screen the ligands on the basis of one or more binding characteristics. Ligands having the first binding characteristic bind to the target of interest thereby to form a target/ligand complex. The complex then optionally is separated from the unbound components using any of a variety of separation techniques, e.g., size exclusion. At least one of the complex or unbound components then is introduced to a second "dimension". The second dimension is capable of separating components based upon a second binding characteristic. One then elutes the ligand having the desired binding characteristics.



INTERNATIONAL SEARCH REPORT

national Application No
PCT/US 96/10929

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N30/88

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K B01D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 15951 A (MATTIASSON) 21 July 1994 see page 1, line 29 - page 2, line 7 see page 17, line 11 - page 18, line 15 ---	1,14,21, 26,28, 29,31, 33,37,47
A	US 5 252 216 A (FOLENA-WASSERMANN) 12 October 1993 see column 2, line 50 - column 3, line 24 ---	1,14,21, 26,28, 29,31, 33,37,47
A	EP 0 411 503 A (TECNOGEN) 6 February 1991 see page 9, line 41-53 ---	1,14,21, 26,28, 29,31, 33,37,47

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[X] Further documents are listed in the continuation of box C.

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1

Date of the actual completion of the international search	Date of mailing of the international search report
22 January 1997	29. 01. 97

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Information on patent family members

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